Supplementary Information

Protecting linear DNA templates in cell-free expression systems from diverse bacteria

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This PDF file includes:

Materials and Methods

Supplementary Tables S1 to S4

Supplementary Figures S1 to S4

MATERIALS AND METHODS

Cell-free lysate preparation

Cell-free lysates were prepared based on our previous work in Yim et al., *Molecular Systems Biology* **15**, e8875, (2019). Strain information and growth conditions used for each species can be found in **Supplementary Table 1**. Briefly, single colonies of each species were inoculated into 4 mL liquid media and grown overnight. 3.3 mL from the overnight culture was transferred to two flasks containing 330 mL growth medium in 1-L flasks and grown to mid-exponential growth phase. Flasks were rapidly chilled on ice and cells were washed three times with 50 mL S30A buffer. The mass of the final pellet was measured and 0.8 mL S30A buffer was added per gram of pellet mass. The resuspensions were sonicated on ice using a Qsonica125 sonicator. Lysates were centrifuged at 12,000 x g for 10 min at 4 ºC. Supernatant was transferred to 2-mL microtubes and run-off reactions were performed by incubating clarified lysates at growth temperature. Samples were centrifuged at 12,000 x g for 10 min at 4 ºC, and supernatant was dialyzed in S30B buffer in dialysis cassette (Slide-A-Lyzer 10k MWCO, Thermo Scientific) for 2-3 h at 4 ºC. Samples were centrifuged at 12,000 x g for 10 min at 4 ºC, then supernatant was aliquoted and stored at -80 ºC.

Cell-free transcription reactions

Cell-free transcription reactions were performed based on our previous work in Yim et al., *Molecular Systems Biology* **15**, e8875, (2019) with minor modifications to use linear DNA template. Briefly, Cell lysates were combined with amino acids, PEG, energy buffer, Mg-glutamate and K-glutamate at concentrations that were previously optimized (Yim et al., *Molecular Systems Biology* **15**, e8875, (2019)) in a skirted white 96-well PCR plate (Bio-rad). A Broccoli expression cassette from TOPO-F30-Broccoli plasmid was used as a DNA template either as a plasmid or as a linear PCR product, and nuclease-free water was used as a negative control. 1 µL of exonuclease inhibitors (GamS, Chi, and Ku) were added at a final concentration of 2 µM, unless otherwise stated. Sequence information of the exonuclease inhibitors can be found in **Supplementary Table 2**. DNA template (1 µL) and 10 mM DFHBI-1T (0.5 µL, Tocris Bioscience) was added to each well immediately before time course measurements. Fluorescence was tracked for 3 h using a Synergy H1 plate reader (BioTek) at 30 ºC using excitation and emission wavelengths of 482 and 505 nm respectively.

Production and purification of Ku in *E. coli*

ku gene from *Mycobacterium tuberculosis* was synthesized (IDT) and cloned into pET28c with riboJ (BBa_K1679038) and RBS (BBa_B0034) to yield pET28c-Ku. The pET28c-Ku plasmid was introduced to *E. coli* BL21(DE3). Ku was produced in the strain by 0.2 mM IPTG (isopropyl-b-D-thiogalactopyranoside) induction at 18 °C for 16 h. Soluble lysate of the

culture was prepared by sonication on ice using a Qsonica125 sonicator with 3.2-mm probe at 40% amplitude for 50 rounds of 30 seconds, with 30 second breaks and clarification by centrifuging at 12,000 x g for 10 min at 4 ºC. Recombinant Ku protein in the soluble lysate was purified using Nickel-NTA-agarose. Ku was recovered in the 300 mM imidazole eluates. Eluted Ku protein was dialyzed in PBS for 2-3 h at 4 ºC, and stored at -20 °C.

Supplementary Table S1. Bacterial species used in this study and their growth conditions

Species	Strain (Source)	Medium	Temp.	Aeration
E. coli	BL21	2xYT+P	37 ℃	Shaking (220 rpm)
S. enterica	Serovar Typhi Ty2	2xYT+P	37 ℃	Shaking (220 rpm)
V. natriegens	ATCC 14048	BHI+v2 salt	37 ℃	Shaking (220 rpm)
B. subtilis	BD3182 (168 derivative)	2xYT+P	30 ℃	Shaking (220 rpm)
C. glutamicum	ATCC 13032	BHI	30 ℃	Shaking (220 rpm)

Supplementary Table S2. Exonuclease inhibitors used in this study

Inhibitor	Sequence	Note
GamS	(AA) MNAYYIQDRLEAQSWARHYQQLAREEKEAELADDMEK GLPQHLFESLCIDHLQRHGASKKSITRAFDDDVEFQERM AEHIRYMVETIAHHQVDIDSEV	Purchased from Arbor Biosciences
Chi	(DNA) TCACTTCACTGCTGGTGGCCACTGCTGGTGGCCACT GCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCC ACTGCTGGTGGCCA	Annealed sense and antisense oligonucleotides
Ku	(AA) MHHHHHHRAIWTGSIAFGLVNVPVKVYSATADHDIRFH QVHAKDNGRIRYKRVCEACGEVVDYRDLARAYESGDG QMVAITDDDIASLPEERSREIEVLEFVPAADVDPMMFDR SYFLEPDSKSSKSYVLLAKTLAETDRMAIVHFTLRNKTRL AALRVKDFGKREVMMVHTLLWPDEIRDPDFPVLDQKVEI KPAELKMAGQVVDSMADDFNPDRYHDTYQEQLQELIDT KLEGGQAFTAEDQPRLLDEPEDVSDLLAKLEASVKARS KANSNVPTPP	Purified using N-terminal 6xHis-tag

Annotated plasmid maps are provided as Supplementary Data

Supplementary Table S3. Plasmids used in this study

Plasmid	Origin	Antibiotic	Description
pTOPO-F30-Broccoli	pUC	Carbenicillin	P_{Gen_18145}-F30-Broccoli-T_{B0015}*
pTXTL-P70a-deGFP	pBR322	Carbenicillin	P_{70a}-deGFP-T_{T500}**
pET28c-F30-Broccoli	pBR322	Kanamycin	P_{T7}-F30-Broccoli-T_{T7}***
pET38c-Ku	pBR322	Kanamycine	P_{T7}-Ku-T_{T7}

Annotated plasmid maps are provided as Supplementary Data

*[Filonov et al., 2014. *J Am Chem Soc* **136**, 16299-308]

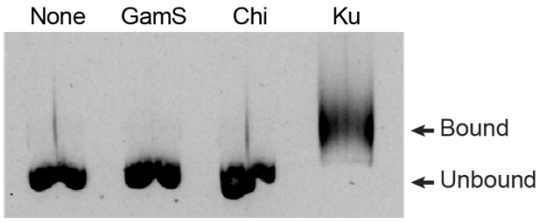
[Sun et al., 2013. *J Vis Exp* **79, e50762]

***[Filonov et al., 2015. *Chem Biol* **22,** 649-660]

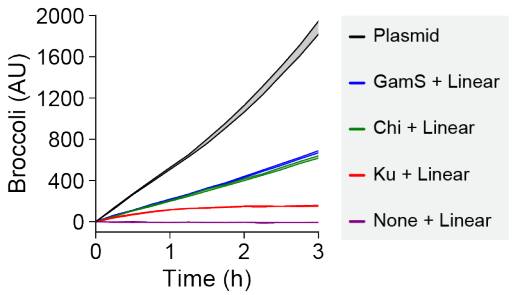
Supplementary Table S4. DNA parts used in this study

DNA parts	Sequence	Note
P_{Gen_18145}	ATTGGGGGCGACGGTCAATTAAGTCCAGTC ATAGTGGGACTGGTGTCCTAATAATTTAAGC ATTCTTTTCTAGAAATACTTTGTCTTTCCTTG ACTTTCATCAGGGTGGTTGTTATATTAGTAA T	Promoter
P_{70a}	TGAGCTAACACCGTGCGTGTTGACAATTTTA CCTCTGGCGGTGATAATGGTTGCA	Promoter
P_{T7}	TAATACGACTCACTATAGGG	Promoter
T_{B0015}	CCAGGCATCAAATAAAACGAAAGGCTCAGT CGAAAGACTGGGCCTTTCGTTTTATCTGTTG TTTGTCGGTGAACGCTCTCTACTAGAGTCA CACTGGCTCACCTTCGGGTGGGCCTTTCTG CGTTTATA	Terminator
T_{T500}	CAAAGCCCGCCGAAAGGCGGGCTTTTCTGT	Terminator
T_{T7}	CTAGCATAACCCCTTGGGGCCTCTAAACGG GTCTTGAGGGGTTTTTTG	Terminator

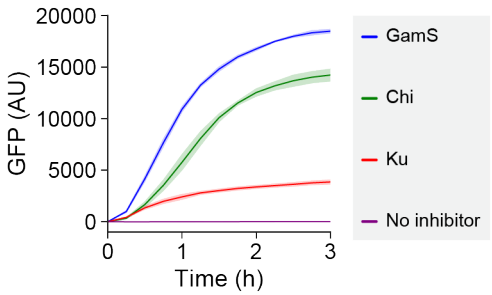
Annotated plasmid maps with these sequences are provided as Supplementary Data



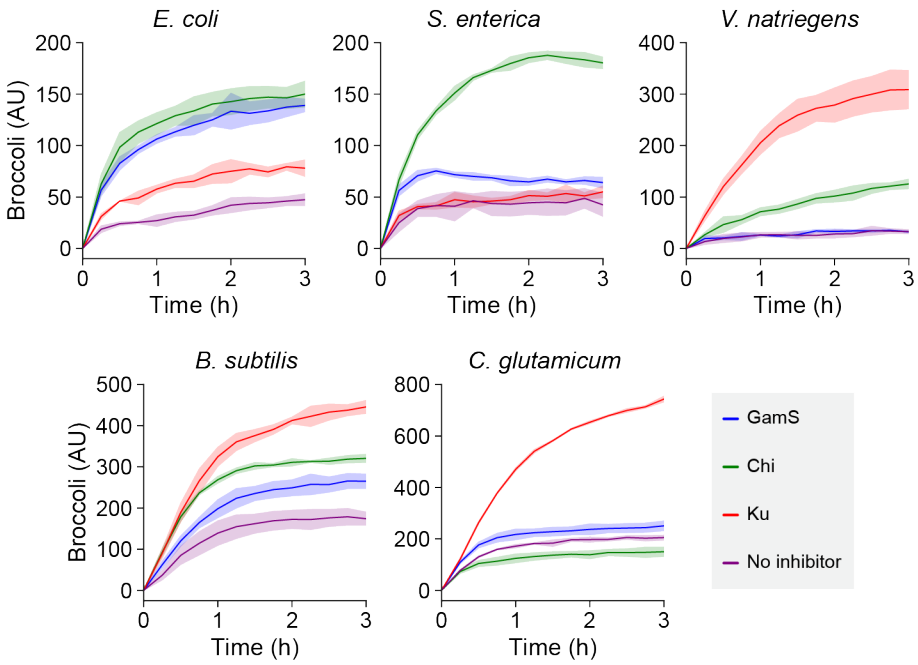
Supplementary Figure S1. DNA binding activity of Ku from *Mycobacterium tuberculosis*. A linear DNA (100 nM) was incubated with each of the nuclease complex inhibitors (1 μM) at 30 °C for 5 min, and interactions between them were analyzed by agarose gel electrophoretic mobility shift assay. The shifted band at the Ku lane indicates Ku from *M. tuberculosis* directly interacts with linear DNA templates.



Supplementary Figure S2. Transcription from plasmid DNA and linear DNA in *E. coli* cell-free expression system. 12 nM of plasmid or linear DNA templates encoding RNA fluorescence aptamer Broccoli under the control of a strong broad-host-range constitutive promoter (P_{Gen_18145}) was used to quantify the level of protection from exonuclease activity using excitation and emission wavelengths of 482 and 505 nm, respectively. 2 μM of each nuclease inhibitor was used. Background signals at 0 h time point were subtracted for normalization. All measurements are based on two biological replicates. Line represents each biological replicate, and shaded region represents range between the two biological replicates.



Supplementary Figure S3. Translation from linear DNA templates using the nuclease complex inhibitors in *E. coli* cell-free expression system. A linear DNA template (12 nM) encoding GFP under the control of a strong sigma70-dependent constitutive promoter (P_{70a}) was used to quantify the level of protection from exonuclease activity using excitation and emission wavelengths of 482 and 505 nm, respectively. Background signals at 0 h time point were subtracted for normalization. All measurements are based on three biological replicates, and shaded region represents standard deviation of the three biological replicates.



Supplementary Figure S4. T7-based assessment of linear DNA stability in diverse cell-free systems. *in vitro* T7-based transcription assay was performed using T7 RNAP (NEB M0251), 1x RNAPol Reaction Buffer, 1.25 mM NTPs, and 12 nM linear DNA template encoding RNA fluorescence aptamer Broccoli under the control of a T7 promoter. RNA fluorescence aptamer Broccoli was used to quantify the level of protection from exonuclease activity using excitation and emission wavelengths of 482 and 505 nm, respectively. 2 μM of each nuclease inhibitor was used. Cell lysates prepared from each bacterial species were added into the T7 reactions at a final concentration of 1 mg/mL. All measurements are based on three biological replicates, and shaded region represents standard deviation of the three biological replicates.